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Gene Therapy for Alpha-1-Antitrypsin Deficiency Diseases

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1. Introduction

Alpha-1-antitrypsin (AAT) deficiency was first described in 1963 by Laurell and Eriksson (Laurell & Eriksson, 1963). They reported an absence of the alpha-1-band in electrophoresis. Alpha -1 -antitrypsin deficiency is one of the most common lethal hereditary disorder of Caucasians of European descent. Alpha -1- antitrypsin also known as α-1-proteinase inhibitor is an archetypal member of the serine proteinase inhibitors, "serpins". The function of alpha -1- antitrypsin is to protect the lower respiratory tract of lungs from proteolytic attack by neutrophil elastase (NE) (Carrell et al., 1982; Brantly et al., 1988). The low circulating levels of AAT expose the lungs to uncontrolled proteolytic attack and predispose the Z homozygous to early-onset panlobular emphysema (Brantly et al., 1988) and liver diseases (Sharp et al., 1969; Sveger, 1976; Eriksson et al., 1986). AAT is synthesized primarily by hepatocytes (Koj et al., 1978; Eriksson et al., 1978) and also by other cells such as monocytes, macrophages, breast carcinoma cells and cornea (Boskovic & Twing, 1998; Geboes et al., 1982; Perlmutter et al., 1985; Ray et al., 1977). In addition, there is a local production of AAT within the lungs by alveolar macrophages and epithelial cells. AAT is present in the plasma at concentrations of 190-280 mg/dl. AAT is a single chain glycoprotein of molecular weight 52kDa containing 394 amino acid residues with the active site residue methionine located at amino acid position 358. AAT also contains three oligosaccharide chains linked to Asn 46, Asn83 and Asn247 respectively. AAT is encoded at the PI locus on chromosome 14 (14q24.3-q32.1) (Cox, 1982, 1985; Schroeder et al., 1985). The PI locus is 12.2 kb in length with 4 coding exons (II, III, IV, and V), 3 noncoding exons (IA, IB, IC) and 6 introns; the region coding for the reactive site loop is located in exon V. AAT shows co-dominant pattern of inheritance (Eriksson, 1965). Accumulating evidence shows that AAT may also exhibit anti-inflammatory activities independent of its protease inhibitor function (Dabbagh et al., 2001; Jeannin et al., 1998; Ikari et al., 2001; Weiss et al., 1993; Bucurenci et al., 1992). For example AAT has been shown to stimulate fibroblast proliferation and procollagen synthesis (Dabbagh et al., 2001) and up-regulate human B cell differentiation into IgE-and IgG4-secreting cells (Jeannin et al., 1998). AAT also inhibits neutrophil superoxide production (Bucurenci et al., 1992), induces the release of macrophage derived interleukin-1 receptor antagonist (Churg et al., 2001) and reduces bacterial endotoxin and TNFα lethal effect *in vivo* (Jie et al., 2003; Libert et al., 1996). AAT also increases the expression of cytokine IL-10 (Janciauskiene et al., 2007). A novel finding indicates that AAT protects the insulin secreting β-cells of pancreas from apoptosis (Zhang et al.,2007).

AAT consists of three β sheets (A-C) , nine helices (A-I) and a mobile reactive site loop that presents the peptide sequence as a pseudosubstrate for target proteinase (Elliott et al., 1996a, 1998, 2000; Ryu et al., 1996; Kim et al., 2001). The active site, Met 358-Ser359, acts as a bait for neutrophil elastase (Johnson & Travis, 1978). After docking, the serine proteinase cleaves the P1-P1'(Met 358-Ser359) of AAT (Wilczynska et al., 1995) and the proteinase is then inactivated by mousetrap action that swings it from upper to lower pole of the inhibitor in association with the insertion of reactive site loop as an extra strand (s4A) in β-sheet A (Wilczynska et al., 1997; Stratikos & Gettins, 1997, 1998,1999; Huntington et al., 2000). This process is accompanied by loss of secondary structure in the serine proteinase and also dramatic rearrangements of the active site. Distortion around the active site of proteinase moves the loop containing the catalytic serine 6A° away from the catalytic histidine residue and thereby disrupts the oxyanion hole (Huntington et al., 2000). These effects results in the inhibition of deacylation reaction. However, after a very long period of time the inhibitory complex breaks down and the active proteinase is released from the complex (Plotnick et al., 2002).

AAT is a highly pleomorphic gene with approximately 125 single nucleotide polymorphisms (SNPs) (Wood & Stockley, 2007) in which a proportion of variants show altered AAT levels or function. These polymorphic variants occur due to amino acid substitutions/deletions which results in charge differences. Based on charge differences, these variants have been identified by isoelectric focusing in the pH range of 4.0-5.0. The most anodal variant is termed as "B" and the most cathodal variant as "Z" (Brantly, 1992; Cox, 2001). The normal variants migrate in the middle region in an isoelectric focusing; hence they are termed as "M". Although, approximately 50 normal AAT variants have been described (Boskovic & Twing, 1998) of these only four normal variants namely M1(Val213), M1(Ala213), M2 and M3 are relatively common with an allelic frequencies among Europeans of Northern European descent of greater than 95% (Kueppers & Christopherson , 1992; Dykes et al.,1984). The M1 (Val213) allele is the most common (allelic frequency 44%-49%) followed by M1 (Ala213) (20%-23%), M2 (14-19%) and M3 (10%-11%) (Kueppers & Christopherson, 1992; Dykes et al., 1984; Nukiwa et al., 1987a). The phylogenetic tree of four of these variants shows that M3 variant is an ancestral protein (Salahuddin, 2010). Variants may also be classified based upon their effect on AAT levels and function. These are normal, deficient, null (nil detectable) or dysfunctional. Deficient variants are susceptible to lungs or liver diseases, whilst null alleles show only lungs diseases. Dysfunctional alleles which while present at a detectable level do not function normally such as the F variant. The majority of clinical diseases of AAT occur due to deficiency and null alleles.

Since AAT molecule has a Met358 residue at its active site, therefore it is readily oxidized by cigarette smoke and thereby resulting in recruitment of inflammatory cells to the lungs. Oxidation of Met358 causes in decrease in association rate constant of AAT for NE by more than 1,000-fold (Travis, 1988).Therefore, cigarette smoking in Z homozygous patients renders an already poorly defended lungs completely defenseless. One unanswered question relating to the pathogenesis of the emphysema is variation in the extent of the disease in individuals matched by age, AAT serum levels, AAT phenotype, and smoking history. This possibly occurred due to variations in the genetic expression of the NE gene.

The Z variant is a more common deficiency variant in Northern Europe whereas S variant is more common in South-West Europe. In Z AAT deficiency variant there is a single base substitution (GAG →AAG) in the codon for residue 342. This causes in amino acid substitution of Glu342 by Lys342 (Jeppsson, 1976). In native state the Glu 342 forms salt

bridge with Lys 290. This salt bridge maintains stable closure of β-sheet A. Mutation of Glu 342 to Lys342 disrupts the structure of β-sheet A. Thus, the β-sheet A undergoes expansion and easily accepts reactive site loop of neighbouring AAT molecule as β-strand and consequently polymers of Z AAT are formed. These polymers tangle in the endoplasmic reticulum of the liver to form inclusion bodies (Lomas et al., 1992; Skinner et al., 1998). The accumulated polymerized protein is retained in the periportal cells of the liver as diastase-resistant periodicacid/ schiff positive inclusions. The polymerized insoluble protein present in the endoplasmic reticulum of hepatocytes eventually causes neonatal hepatitis, juvenile cirrhosis and hepatocellular carcinoma (Geboes et al., 1982; Perlmutter et al., 1985; Ray et al., 1977). Owing to the polymerization in the ER of hepatocytes, the AAT is not secreted into the blood stream therefore the levels of circulating AAT in plasma decrease to <11 μM or <570 μg/ml and are unable to protect the lungs from proteolytic degradation. Thus, Z AAT homozygous is predisposed to develop early onset panlobular emphysema (Boskovic & Twing, 1998). Only a subset of patients with AAT deficiency experience liver disease. In these patients a cascade of aberrant signals are also triggered within the hepatocyte, most likely due to the result of an unfolded protein response. However, the downstream details remain unclear. The mode of polymerization seen in ZAAT also underlies the Mmalton (52Phe deleted) (Matsunaga et al., 1990) and Siiyama (Ser53Phe) variants of AAT (Seyama et al., 1991, 1995; Lomas et al., 1993).The S variant (Glu264Val) shows a slower rate of polymer formation because the structural changes in β -sheet A are not substantial (Elliott et al., 1996b; Mahadeva et al., 1999) resulting in a milder form of serum deficiency with no clinical consequences. If an individual has the genotype PiSZ, then their clinical phenotype for liver disease (Mahadeva et al., 1999) and lung disease in smokers (Turino et al., 1996) is intermediate between that of PiZ and PiS subjects. There are many null-allelic variants of AAT that are absent in plasma, and are termed as QO rather than Pi. In QOgranite falls genotype there is deletion of a single base pair that leads to the premature stop codon and unstable mRNA (Nukiwa et al., 1987b). Likewise there is deletion of 2 bp in exon IV in QOhong kong causing in a premature stop codon, and unstable mRNA hence a truncated protein (Sifers et al., 1988). Thus, both QO granite falls and QO hong kong show accumulation in the endoplasmic reticulum of the liver resulting in plasma deficiency. Similarly, an amino acid substitution at the active site (Met358Arg), in dysfunctional Pittsburgh AAT results in reduced neutrophil elastase inhibitory activity as well as inhibition of factor IXa, kallikrein and factor XIIf (Scott et al., 1986)

Gene therapy is a therapeutic strategy in which genetic material, in the form of cDNA /RNA is transferred to an individual to correct a hereditary disorder or to treat and/or prevent an acquired disease (Anderson, 1992; Miller,1992; Crystal,1992; Mulligan, 1993). Gene therapy depends upon vectors for carrying normal gene. These vectors integrate with host genome and all cells resulting from cell division of the host cell will contain the copy of the correct gene in place of the defective one. Gene therapy can be classified into three distinct classes: 1) gene addition also known as gene replacement, ii) gene reprogramming and iii) gene repair. Gene addition is currently the most popular technique which is clinically tested. Gene addition is used for curing diseases that occur due to loss-of-function mutations. This technique involves delivery of a corrected copy of the defective gene without removal of the endogenous mutated gene. Advantages of gene addition lie in its simplicity, whereas a disadvantage is lack of regulated gene expression in some cases. For a less complex diseases, gene replacement strategies are most suitable. However, for more complex diseases in which the gene product requires regulated gene expression, gene

reprograming is more appropriate. The gene reprogramming approach involves inhibition of the expression of mutated gene by modification of messenger RNA (mRNA). Although, this approach also expresses an additional genetic material within cells, the net result is correction (or reprogramming) of endogenous mutant gene. This is in contrast to the expression of additional intact functional gene in gene addition technique. A disadvantage of this technique is inefficient nature of the reprogramming. The third approach namely, gene repair is a more developed technique than gene addition or reprogramming approaches. This technique in contrast to gene addition involves correction of mutant sequences at the genomic DNA level but is limited owing to its low efficiency. There are various types of viral and non-viral vectors. The examples of some viral vectors are retroviruses, adenoviruses and adeno-associated viruses (AAVs) and examples of non-viral vectors is liposomal vector and other means by which it is transported into the cell are naked DNA injection and gold-particle bombardment.

For the treatment of patients with AAT deficiency with impaired lungs function is weekly infusion of AAT protein derived from human plasma (Heresi & Stoller, 2008). However, this method has several disadvantages like high cost, inconvenient route of administration, risks of blood borne diseases and time-consuming nature of the therapy thus provide an impetus to develop alternative treatment modalities such as gene therapy. Indeed a phase I AAT gene therapy clinical trial has been approved at the University Of Florida College Of Medicine, Gainesville, Florida, USA. The trial involves intramuscular injection of AAT gene using a recombinant adeno-associated virus (rAAV) vector and thus provides stable plasma levels (Song et al., 2002). In view of above, in this chapter I have discussed about viral and non-viral vectors in gene therapy, their advantages and disadvantages and their applications in alpha-1-antitrypsin deficiency diseases.

2. Viral vectors for gene therapy

2.1 Retroviruses

Retroviruses (Retroviridae) are enveloped single stranded RNA viruses that have been widely used in gene transfer technique. The retrovirus is reverse transcribed from single-stranded RNA genome into a double stranded DNA, which can integrate into host chromosomes (Fields & Knipe, 1986). The retrovirus genome contains three open reading frames that encode for group specific antigens (gag) that codes for core and structural proteins of virus, similarly, polymerase (pol) codes for reverse transcriptase, protease and integrase, and envelope (env) codes for retroviral coat proteins. All of the retroviral genomes have packaging signal Ψ and *cis* acting sequences known as long terminal repeat (LTR) present at each ends. These LTR and neighboring sequences act *in cis* during viral gene expression, and packaging, retro-transcription and integration of the genome (Figure 1). The common example of retroviral vector is Moloney Murine Leukaemia Virus (Mo-MLV). This virus has varying cellular tropisms depending upon the receptor binding domain of envelope glycoprotein. Retroviral vectors upon binding to the host cell receptor undergo conformational changes within envelope glycoprotein leading to their fusion with the host cell membrane and thereby release capsid core into the cytoplasm. Once inside the cytoplasm the single stranded RNA genome is reverse transcribed into double stranded DNA proviral genome by an enzyme reverse transcriptase. The proviral genome subsequently forms a preintegration complex with the viral integrase and thereby it is transported to the nuclear membrane. The pre-integration complex enters the nucleus during mitosis as nuclear membrane is disrupted (Roe et al., 1993).

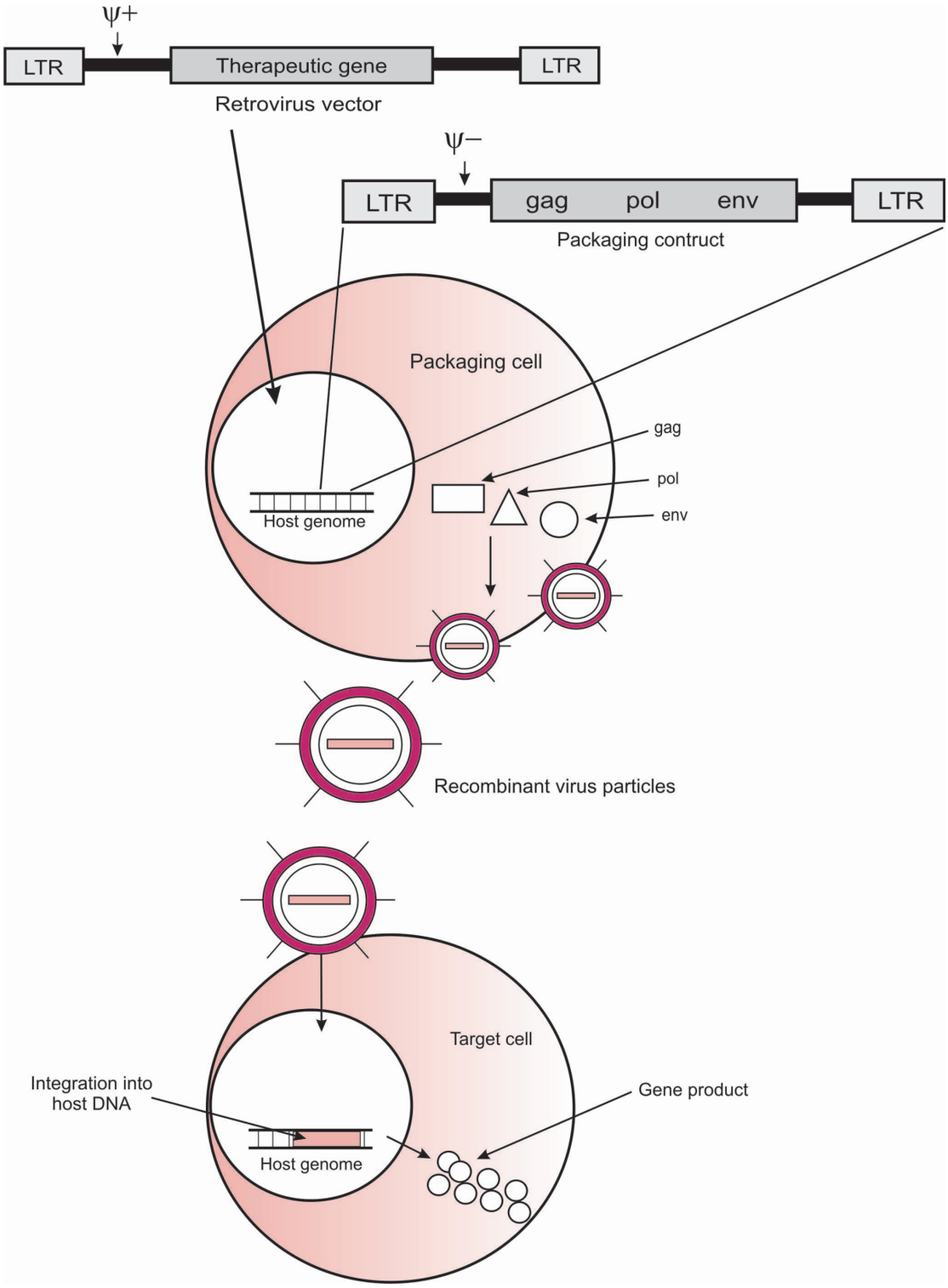

Fig. 1. Structure of retroviral and retroviral gene transfer and production. The *gag, pol* and *env* genes required for viral production are integrated into the packaging cells genome. The vector provides the viral packaging signal, commonly denoted Ψ, and a target gene

After entering the nucleus, the viral integrase randomly integrates the proviral genome into the host chromosomal genome and consequently the viral genes are expressed by host replication machinery. Retroviral vectors are created by removal of the operon like retroviral *gag*, *pol* and *env* genes. These genes are replaced by therapeutic gene of interest (Figure.1). Up to eight kb of exogenous DNA can be inserted and expressed in place of the viral gene. The LTRs and Ψ sequence are the only viral sequences that are present in vector, and thus this vector is insufficient to express the viral proteins. To express the viral proteins, it is necessary to supply the viral genes *gag*, *pol* and *env in trans* (Figure. 1). This can be achieved by creating packaging cell lines that express these genes. Removal of *gag, pol* and *env* genes *in vivo* reduce immune responses against the virus. The tropism of retroviruses can be modified by removing the native envelope protein and providing an alternative envelope glycoprotein *in trans* during virus production. This process is known as pseudotyping of virus. The pseudotyping of retrovirus thus provides broader tropism and enhanced stability upon concentration (Kang et al., 2002; Stein, et al., 2005; Wong et al., 2004). One problem encountered in gene therapy using retroviruses is that the integrase enzyme can insert genetic material of the virus at random position in the genome of the host. If genetic material is inserted in the middle of gene of the host cell, this gene will be disrupted causing insertional mutagenesis. If the gene is inserted during regulation of cell division, an uncontrolled division may occur like cancer or inactivation of tumour supressor gene. This problem has recently been solved by using zinc finger nucleases (Durai et al., 2005) or by including certain sequences such as the beta-globin locus control region to direct integration to specific chromosomal sites. Other disadvantages include limited insert capacity (8kb), low titer, their inactivation by human complement factor and their inability to transduce non-dividing cell. Their advantages are : ability to transduce dividing cell, inability to express viral proteins that could be immunogenic and their ability to achieve long term transgene expression.

2.2 Adenoviruses

Adenoviruses are medium-sized (90-100nm) non-enveloped icosahederal viruses composed of nucleocapsid and double-stranded linear DNA (Fields & Knipe, 1986). There are over 51 different serotypes in humans, which are responsible for 5-10% of upper respiratory infection in children and several types of infections in adult as well. They efficiently infect and express their genes in wide variety of cell types including dividing and non-dividing cell. The adenovirus has inverted terminal repeat (ITR) (Figure. 2) sequence at its both ends, and the gene transcript can be divided into two distinct phases: early genes (E) expressed before the onset of viral DNA replication and late genes (L) expressed after the onset of viral replication. One of the essential proteins in the viral replication process is the E1A gene product. The E1A is the first gene to be expressed after infection and has a keyrole as transactivator of all other adenoviral genes. Viral infection is mediated through binding of fiber knob protein of the virus to the primary Coxsackie-Adenovirus Receptors (CAR) (Bergelson et al., 1997) present on the cell surfaces. This is followed by interaction between the cell surface integrins alpha v beta 3 and alpha v beta 5 and virion penton base (Wickham et al., 1993). Consequently, the adenovirion is endocytosed through clathrin-coated pit into an endosome. After this the pH of endosome decreases leading to conformational changes in varion capsid proteins which culminate in the release of viral capsid into the cytoplasm.

The viral capsid translocates from cytoplasm to nucleus of cell where viral genome is released and undergoes replication and transcription as an extrachromosomal element (Figure 2). Adenoviruses infects a variety of quiescent and proliferating cells in different species and can mediate normal gene expression. The transgene expression is mostly transient due to host immune responses (Yang et al., 1996; Stein et al., 1998). The adenoviral vectors have been shown to give therapeutic levels of human alpha-1-antitrypsin (Kay et al., 1995).

First generation recombinant adenovirus vectors (rADs) are generally derived from the human adenovirus serotypes 2 and 5 and these vectors are rendered replication-defective through deletion of the E1-region. Large foreign DNA is inserted in place of E1 deleted region of adenoviral genome (Figure.2). These rADs are propagated in cell line expressing E1 segment (Figure 2). For insertion of larger transgenes, first generation rADs may also carry deletions in the E3 region (Bett et al. , 1994). Several such cell lines like HEK 293(Graham et al.,1977) and 911(Falloux et al., 1996) have been developed that are cable to propagating replication defective rADs vector.

Recently, new varieties of rADs vectors have been developed that are less immunogenic than first generation rADs vectors. These vectors lack or are defective in the E2, E3 or E4 regions and can be propagated in trans-complementing cell lines (Englehardt et al., 1994a, b; Yang et al .,1994; Brough et al., 1996).Additionally, there are vectors called gutless vectors that are devoid of all viral genes and contain only ITR and packaging signal of wild type virus. These gutless vectors can carry up to 35kb of foreign DNA (Kochanek et al., 1996; Parks et al., 1996; Schneider et al., 1998). In order to propagate, these gutless vectors need helper virus containing all genes for virus assembly *in trans* (Parks et al., 1996). Gutless adenoviruses have been shown to give rise to sustain transgene expression compared to first generation adenoviruses (Morsy et al., 1998).

2.3 Adeno-associated viruses

Adeno-associated Viruses (AAVs) belong to the family Parvoviridae. AAVs are small single-stranded DNA genome containing inverted terminal repeats (ITRs) at both ends of the DNA strands and contains two open reading frames (ORFs): rep and cap. The rep is composed of four overlapping genes encoding rep proteins required for the AAV life cycle and the cap contains overlapping nucleotide sequences of capsid proteins namely VP1, VP2 and VP3. These capsid proteins interact together to form a capsid of icosahederal symmetry. The ITRs are the only *cis* acting elements required for efficient encapsidation and integration of the viral genome into the genome of the host cells (Samulski et al., 1989). The wild-type AAV is non-pathogenic. AAVs infects broad host range. The AAVs genome integrates into specific locus on human chromosome 19 (Kotin & Berns, 1989). The AAV vectors can transduce both mitototic and post mitototic cells (Summerford & Samulski, 1998; Summerford et al., 1999). The AAV does not integrate into the genome rather it transduces cells and expresses the transgene as an episome. Disadvantages of AAV vector is that it is unable to replicate unless sequences are provided *in trans* by a helper virus such as adenovirus and HSV. Other disadvantage of this vector is limited packaging capacity for transgene (4.7 kb). These adenovirus (Schiedner et al., 1998; Morral, et al., 1998) and rAAV vectors have been shown to achieve stable levels of alpha-1-antitrypsin greater than 800 µg/ml.

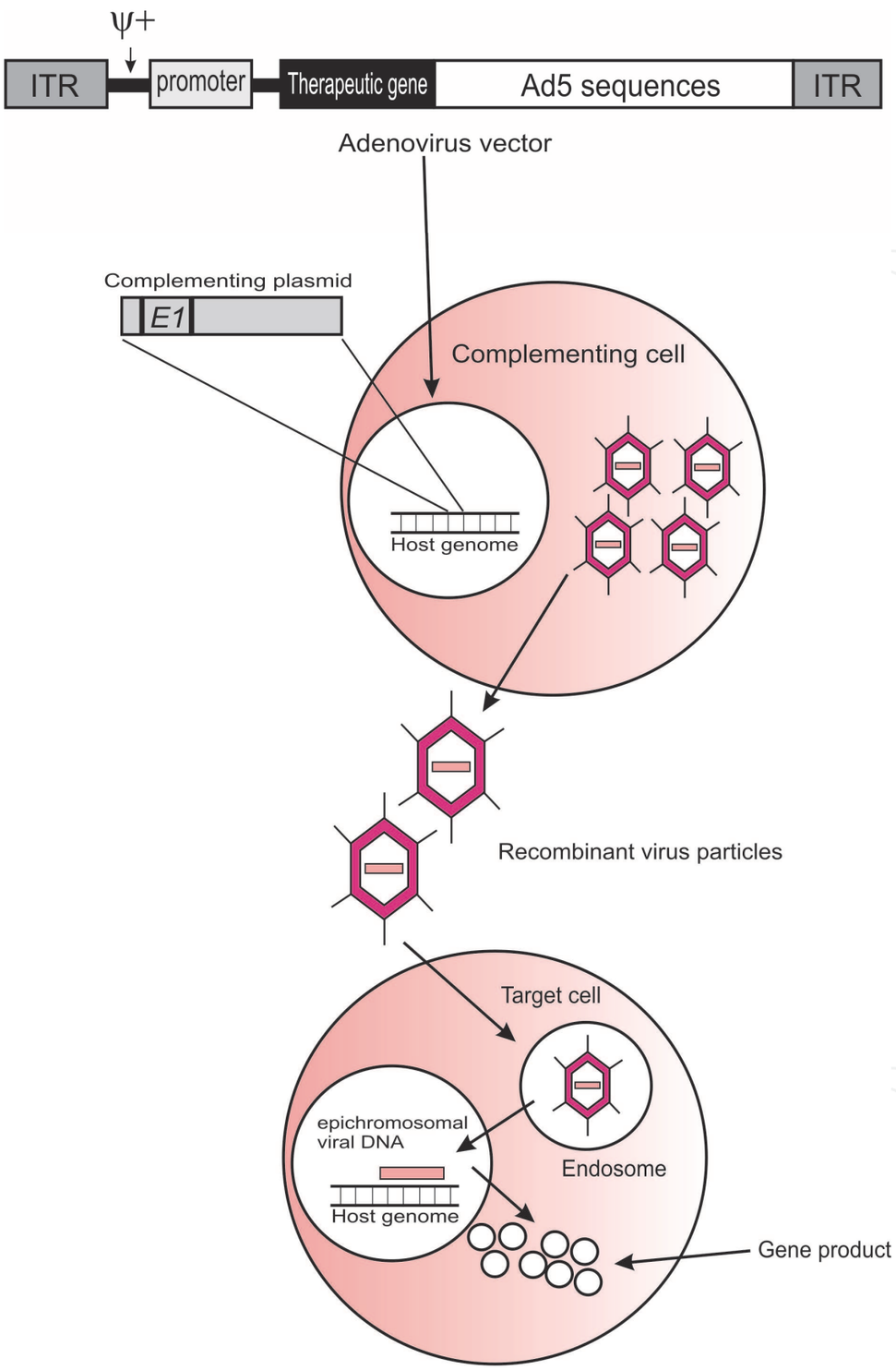

Fig. 2. Structure of adenoviral vector and principle of adenovirus production. Adenovirus vectors are based on serotypes 2 and 5. Therapeutic genes are placed into the deleted E1 region of the viral genome, driven by internal promoters. The function of E1 for production of viral particles is provided by the complementing cell line expressing E1.

3. Non-viral vectors for gene therapy

Non-viral vectors for gene delivery has several advantages over viral vectors. Viruses cause an immune response that can make repeated administrations ineffective. Non-viral vectors on the other hand do not elicit a specific immune response. Non-viral vectors do not randomly integrate DNA into the genomic DNA of the host. Nonviral vectors can also carry more DNA than viral vectors, allowing the delivery of larger genes. In addition, nonviral vectors are easier and less expensive to manufacture. Moreover, vector like plasmids are non-pathogenic and therefore they carry less risk to harm than most viral vectors. Disadvantage of using nonviral vectors for gene therapy are its low efficiency of transgene expression and short duration of expression. Non-viral vectors can be divided into two distinct categories: physical and chemical. Physical methods involve forcing the plasmid into cells through electroporation, sonoporation, or particle bombardment. Chemical methods employ lipids, polymers, or proteins that will complex with DNA, condensing it into particles and directing it to the cells. Many types of DNA deliveries have been proposed. They are i) naked DNA ii) cationic lipids iii) cationic polymers.

3.1 Naked DNA

Naked DNA (in the form of a plasmid) can be transferred by directly injecting into muscle cells (Wolff et al., 1990).Though, it is not very efficient, but can result in prolonged low level expression *in vivo*. The simplicity of this method and sustained expression has led to the development of DNA vaccines. Several approaches have been developed to enhance the efficiency of gene transfer *via* naked DNA including gene gun (Yang et al., 1990) and electroporation (Rols et al., 1998). These physical approaches allow DNA to directly penetrate cell membrane and bypass endosome/lysosome, thus avoiding enzymatic degradation. Further, it has been reported that intramuscular injection of plasmid DNA followed by electroporation results in an impressive level of gene expression (Rizzuto et al., 1999).

3.2 Cationic lipids

Gene transfer can be achieved either by direct intratissue injection of naked plasmid DNA or by intratracheal and intravenous injection which generally requires the use of a delivery vector or vehicle. Various types of synthetic vectors have been developed for gene transfer. Among these, cationic lipid- and polymer-based systems have been the most extensively studied. Cationic lipids are amphiphilic molecules consisting of hydrophilic and hydrophobic regions, i.e., a (charged) cationic (amine) headgroup, attached via a linker (for example glycerol) to a hydrophobic double hydrocarbon chain or a cholesterol derivative. Positively charge head group of cationic lipids and cationic polymers form complexes with negatively charged phosphate backbone of DNA through electrostatic interactions thereby it spontaneously form compact structures called lipoplexes. The cationic lipid-DNA complexes are protected from extracellular or intracellular nucleases. Furthermore, positive charges of lipolexes, tend to electrostatically interact with the negatively charged molecules of the cell membrane. This may facilitate their cellular uptake. Transfection efficiency of cationic lipids depend on the structure of cationic lipids (the geometric shape, the number of charged groups per molecules, the nature of lipid anchor, and linker bondages), the charge ratio used to form DNA–lipid complexes, the size of DNA, conformation of DNA and the properties of the colipid (Wasungu & Hoekstra, 2006). These colipids are mostly cholesterol and dioleoylphosphatidylethanolamine (DOPE). The role of these colipids in cationic

liposome-mediated gene transfer depends upon the structure of cationic lipid. Some cationic lipids require DOPE for a normal level of transfection, while other cationic lipids like double fatty chains are capable of forming bilayer or micellar structures that do not depend upon helper lipid for transfection. Most cationic lipids are more or less toxic to cells, and inclusion of colipid DOPE reduce the charge ratio thereby reducing toxicity.

3.3 Cationic polymers

Cationic polymers have also been extensively used for gene transfer. When cationic polymers are mixed with DNA, they form nanosized complexes that are known as polyplexes. These polyplexes are more stable than lipoplexes. Among cationic polymers, PEI is most effective polymer used for transfection. PEI was first used in gene transfer in 1995 (Boussif et al., 1995). These PEI exist as either linear or branched polymer. PEI contains high content of amine groups most of which are nonprotonated at the physiological pH. These nonprotonated amines exert buffering effect, which effectively stop the acidification of the endosomal pH by neutralizing the protons that are pumped by membrane transporter, ATPase (Akinc et al., 2005; Yamashiro et al., 1983). This eventually leads to influx of chloride counter ions within the compartment and thereby osmotic pressure develops that causes the swelling and breakdown of the endosomal membrane. Transfection capacity and toxic nature of PEI depends on its molecular weight (MW), configuration, and the charge ratio of polymer to DNA used. Several studies have demonstrated that high molecular weight PEI (greater than 25,000 Da) is toxic and less efficient in transfection while polymers prepared from medium to low MW (5,000–25,000 Da) are less toxic and more efficient in transfection (Fischer et al., 1999). Besides this, branched PEI shows high toxicity and low transfection efficiency compared to polyplexes prepared from linear chain of PEI (Wightman et al., 2001).

4. Gene therapy applications in alpha-1 -antitrypsin deficiency diseases

The deficiency of AAT in plasma causes reduced protection against neutrophil elastase in lungs, this eventually leads to emphysema. A logical approach to treat this disease is to raise the levels of AAT in plasma to above 11μM (570–800 μg/mL; 57–80 mg/dL). One specific treatment for AAT deficiency available at present is augmentation therapy which involves administration of plasma purified AAT intravenously. According to Hubbard and Crystal (1990), approximately only 2–3% of the infused AAT actually reach the lungs. Therefore, alternative routes of administration, such as inhalation of nebulized AAT powder or aerosolized AAT solution (Hubbard et al., 1989; Hubbard & Crystal, 1990; Sandhaus, 2004; Taylor & Gumbleton, 2004), provided the protection against lungs diseases. However, for treating lungs and liver diseases alternative therapy namely gene therapy provides long term solution (Flotte, 2002; Stecenko & Brigham, 2003; Sandhaus, 2004). Several vectors containing cDNA of AAT have been constructed for treating AAT deficiency diseases. These vectors are retroviral (Kay et al., 1992), adenoviral (Jaffe et al., 1992; Rosenfeld et al., 1991; Morral et al., 1998; Schiedner et al., 1998; Kay et al., 1995) and adeno-associated viral (Lu et al., 2006; Song et al.,1998;2001) Besides this, AAT gene can also be transferred by liposomal vectors (Alino et al., 1994; Canonico et al., 1994), naked DNA injection and gold-particle bombardment (Qiu et al., 1996).

First clinical trial has demonstrated that AAT gene could be transferred in humans (Brigham et al., 2000). Patients with AAT deficiency received a single dose of non-viral cationic liposome and AAT complex into one nostril, while other nostril acts as a control. Protein

was detected in nasal lavage fluid, with maximum levels on fifth day, which is approximately one third of the normal levels. This rise in AAT was not seen in fluid from the control nostril. Besides this, levels of the pro-inflammatory cytokine, IL-8, was decreased in the treated nostril. Surprisingly, when purified AAT protein was intravenously administered, the decrease in pro-inflammatory cytokine, IL-8, levels was not observed in normal nasal implying that different routes of administrationmay lead to variations in the production level of IL-8.

The retroviral vector containing cDNA of human AAT with constitutive promoter have also been used as a delivery system. After packaging into an infectious virus, the provirus was integrated into murine fibroblasts and it expressed and secreted human AAT. This AAT was found to be glycosylated, reacted normally with human NE, and had a normal half-life in plasma. When these cells were transplanted into the peritoneal cavity of nude mice, one month latter human AAT was present in plasma and most notably it was present in lung epithelial lining fluid (Garver et al., 1987).Thus, this vector has a potential to cure lungs disease associated with AAT deficiency. The disadvantage of retroviral vector system is that transgene expression is low. Therefore researchers have to resort to better vector systems like adenovirus and adeno-associated virus.

The adenoviral vectors containing human AAT cDNA have been delivered to different organs and cells. For example replication-deficient adenoviral vector containing an adenovirus major late promoter and a recombinant human AAT gene (Ad-AAT) was infected to epithelial cells of the cotton rat respiratory tract both *in vitro* and *in vivo* (Rosenfeld et al., 1991). Results *in vitro* demonstrated that human alpha-1-antitrypsin was synthesized as well as secreted. Whereas i*n vivo* intratracheal administration of Ad-AAT to these rats, resulted in the synthesis and secretion of human AAT by lung tissue, and AAT was detected in the epithelial lining fluid for at least 1 week. This mode of administration of Ad-AAT has a potential to cure emphysema. Similarly, when replication defective adenovirus vector containing human AAT cDNA was transferred to human endothelial cells *in vitro* and in the lumen of intact human umbilical veins *ex vivo* (Lemarchand et al., 1992), the infection resulted in the expression of AAT transcripts and synthesis and secretion of AAT both in cell culture (0.3-0.6 µg) and in the vein perfusates (13 µg/ml). The therapeutic level of AAT was achieved in vein perfusate. In another study recombinant adenoviral vectors containing human alpha-1-antitrypsin cDNA under the transcriptional control of the phosphoglycerate kinase (PGK) or RSV-LTR promoters was constructed and transduced in mouse hepatocytes *in vivo* (Kay et al., 1995). The therapeutic serum level of human alpha-1-antitrypsin of up to 700 micrograms/ml was achieved *in vivo*. Thus, this vector has a capacity to cure liver and lungs diseases, but it cannot knock down the expression of misfolded Z AAT in liver. The adenoviruses are pathogenic in nature as well as immunogenic, therefore they have limited applications in treating AAT deficiency diseases.

Recombinant adeno-associated viral vectors have been most successful delivery system so far, as they are capable of achieving therapeutic levels of AAT (Lu et al., 2006; Song et al., 1998) and are less likely to induce an inflammatory response than adenoviral vectors. Studies have been conducted to examine delivery of rAAV-AAT vectors to muscle, lung, or liver (Song et al., 1998, 2001; Virella-Lowell et al., 2005; Liqun Wang et al., 2009). Although, maximum expression could be achieved by each delivery route, however the use of muscle injection has several advantages over other approaches , such as a more favorable profile of anti-capsid effector T-cell responses (Manno et al., 2006; Brantly et al., 2009) and a lower

level of dissemination to distant sites (Manno et al., 2003; 2006). A phase I trial of intramuscular injection of a rAAV2-AAT vector has recently been completed but levels of gene expression were found to be very low and generally undetectable. Studies have shown that AAV1 capsid transduction and expression in muscle was substantially more efficient than AAV2 (Xiao et al., 1999; Chao et al., 2001; Gao et al., 2002; Rabinowitz et al., 2002; Hauck & Xiao, 2003). The phase I trial of a rAAV1-AAT vector has been initiated (Brantly et al., 2009.). There are several problem associated with rAAV: firstly scarcity of available AAV2 receptors on the apical surface of airway epithelium, secondly degradation of AAV in the cytoplasm, and thirdly lack of integration of rAAV has limited its efficacy in clinical trials. Several alternative AAV serotypes such as rAAV5, rAAV8, rAAV9, or AAV2 capsid mutants with specifically targeted ligands have been developed. These alternative capsids may bypass the binding and internalization barriers and achieve improved gene expression from a single administration. Among these tested rAAV serotypes, the rAAV8 was found to be more powerful gene therapy vector as it efficiently transduced many different tissues *in vivo* and transduced a high percentage of cells in the lung when delivered intratrachealy (Liqun Wang et al., 2009). It should be noted these approaches whilst potentially protecting the lungs and other tissues, will not influence the liver disease.

The Z AAT variant undergoes polymerization; therefore it accumulates in the ER of hepatocytes. Hence it is associated with liver disease; therefore gene therapy aiming at inhibiting the expression of the Z AAT gene in liver represents a promising therapeutic approach. A number of methods aimed at turning off production of the abnormal Z AAT protein are being considered. These include the use of and siRNA, ribozyme technology, gene repair, PNA and SDF. In a recent report, small-interfering RNAs (siRNAs) was designed to downregulate endogenous Z AAT within hepatocytes. Three different siRNA sequences were cloned into a recombinant adeno-associated virus (rAAV), either singly or as a trifunctional (3X) construct. Each of these vectors demonstrated activity independent of other. These studies showed a decrease in total human Z AAT when rAAV-3X-siRNA packaged into AAV8 capsids was used, thus removing ZAAT accumulation from liver. The rAAV8-3X-siRNA vector may hold promise as a potential therapy for patients with AAT liver disease (Cruz et al., 2007a). Similarly, ribozyme-mediated specific gene replacement also represents a novel mode of gene therapy that aims to treat the AAT deficiency by inhibiting the expression of the mutated gene and at the same time also replacing the defective gene with a normal AAT gene in the liver (Ozaki et al., 1999). Unfortunately, this approach has not been successful todate. A technology called gene repair has been developed which uses chimeric RNA/DNA oligonucleotides to ''patch'' a single gene mutation. RNA complementary to the area surrounding the point mutation is synthesised with a contiguous DNA oligonucleotide made of the corrected sequence. In model systems, chimeraplast constructs were capable of correcting targeted single site gene mutation (Cole-Strauss et al., 1999). Another mode of gene therapy utilizes second- as well as third-generation oligonucleotides based on a peptide nucleic acid (PNA). These PNA are analogs in which the phosphodiester backbone is replaced by repetitive units of the pseudo-peptide polymer N-(2-aminoethyl) glycine to which the purine and pyrimidine bases are attached by a methyl carbonyl linker (Pellestor & Paulasova, 2004). PNAs hybridize to complementary DNA or RNA in a sequence specific manner. These PNAs are more stable and form hybrid with DNA by displacing one strand of DNA thereby inhibiting gene transcription. Similarly, when these PNAs bind to RNA they act as an antisense (McLean et al., 2009). The potential of PNA in treating AAT deficiency diseases remains to be seen. Furthermore, small DNA

fragments (SDFs) have been used for correcting the sequence of variant AAT. This strategy involves small fragment homologous replacement (SFHR) in which the sequences can be directly altered, inserted or deleted. These SDFs can directly convert the mutant sequence to a wild-type genotype, thereby restoring the normal phenotype. Indeed SDFs of normal M and Z AAT sequences were synthesized and transfected into peripheral blood monocytes of PiM subjects and PiZ subjects. The defective gene was corrected in Z AAT monocytes *in vitro* and consequently this treatment was associated with an increase in AAT secretion. This technology has potential to protect both lungs and liver (McLean et al., 2009; McNab et al., 2007). The rAAV2 vectors, such as those used in current clinical trials, appear to be incapable of stably transducing more than 5% of the hepatocyte population. Recently, AAV2 and pseudotyped vectors for serotypes 1, 5, and 8 carrying the human AAT transgene were injected into C57Bl/6 mice. Circulating hAAT level was found to be highest for AAV8 injected animals. Most notably, up to 40% of total liver cells stained positive for the transgene in AAV2/8 subjects. This suggests that different types of AAV use different cellular pathways for infection and AAV8 serotype is a powerful gene therapy vector (Conlon et al., 1982).

The transfection efficacy of several non-viral plasmid vectors containing the full genomic hAAT gene with its natural promoter (pTG7101) and others containing the cDNA of hAAT gene driven by cytomegalovirus immediate-early promoter or the 0.5 kb upstream of hAAT gene sequence has been studied by hydrodynamic tail-vein injection (20 mg/mouse) (Alino et al., 2003).These studies demonstrated that only pTG7101 plasmid results in expression of hAAT in plasma. Further, it was found that hAAT remains long-term stable in plasma, with therapeutic concentrations of hAAT (40.9 mg/ml). In addition, 4 months after transfection, the efficacy of transgenic expression (amount of RNA/DNA) in mouse liver was 50–80% that normally expressed by the mouse gene. Researchers have constructed non-viral plasmid vector which contained AAT gene and eukaryotic replication initiation sequences from Epstein–Barr virus, EBNA1 and from its family of binding sites. Using hydrodynamic injection approach, they found greater than 300 mg/ml of AAT in serum, and increased in serum AAT concentrations occurred for greater than nine months after a single dose of administration of the vector (Stoll et al., 2001). These studies demonstrated that naked plasmid DNA injection is a good technique for curing AAT diseases. Similarly, intravenous injection of linear DNA encoding the AAT gene driven by an RSV promoter into mice is reported to achieve 10–100 fold higher serum AAT concentrations than similar administration of circular DNA with expression persisting for at least 9 months (Chen et al., 2001). Thus, the linear form of the DNA may contribute to more excessive and prolonged *in vivo* expression than the circular DNA.

Studies have shown that lipoplex technology can deliver normal AAT gene to human respiratory epithelium *in vivo* (Brigham et al., 2000). This mode of therapy is safe, but their limitations are similar to those of other nonviral gene therapy. Despite of some progress, the lipid vectors are still inefficient in targeting the genes to distant tissues. Efforts are in way to improve these vectors which include modification of their surface to improve their biocompatibility with biological fluids and tissue specificity, and inclusion of active components that help to overcome the cellular barriers in transfection.

Adult stem cell-based gene therapy holds several unique advantages including avoidance of germline or other undesirable cell transductions. Moreover, stem cells have a capacity for both self-renewal indefinitely and differentiation, making them an ideal vehicle for

permanent delivery of normal genes to those affected by loss-of-function genetic mutations. Research using stem cells have also shown some potential for treatment of AAT disease, though these approaches will require further development before clinical use. Since stem cells can differentiate into liver cells that are capable of expressing AAT (Zhou et al., 2007; Moriya et al., 2007; Saito et al., 2006), thus transplanting these cells into deficient patients might facilitate normal production of AAT. This approach however would not prevent accumulation of misfolded AAT in the liver, therefore it would not necessarily affect liver disease. Alternatively, an approach targeting the lung might be used, in this technique human embryonic stem cells are differentiated into alveolar epithelial type II cells (Wang et al., 2007). This alveolar type II cells capable of producing AAT could be transplanted to AAT deficient patients for producing AAT.

5. Future prospects

To further enhance this transduction of viral vectors researchers have developed a novel AAV vector that packages a double-stranded (ds) genome (Richard, 2011). These vectors by-pass the rate limiting step of second-strand synthesis resulting in both increased and faster transgene expression. In liver, researchers have been able to demonstrate dsAAV 2 transduction is 90% whereas dsAAV 8 transduction is over 95% using a lower dose. Thus, it is evident that suppression of PiZZ gene expression along with the successful gene addition strategy should eliminate both liver and lungs diseases respectively. It should be noted that although AAV2 and AAV8 transduce the liver with high efficiency the overall tropism is broad and is not restricted to liver cells. Several steps including viral binding, endocytosis, trafficking and uncoating are required for cell specific transduction and research are on way to relate structure to function using a shuffled serotype capsid library (Richard, 2011). Thus, transduction by these alternative serotypes vectors could be restricted to liver cells. Additionally, to down-regulate Z-AAT researchers have also begun to examining innovative approach for long-term expression of therapeutic RNAs using the recombinant adeno-associated virus (rAAV) vector, including spliceosome-mediated RNA trans-splicing (SMaRT) (Cruz et al., 2007b). This technology has a potential to correct Z AAT mutation at the molecular level. Recent reports indicate that chemical chaperones, such as 4-phenylbutyric acid (4-PBA), can assist the proper folding of Z AAT (Burrows et al., 2000). This implies that transduction of rAAV-Hsp70 vectors construct into liver cells of animals and humans could increase the folding of mutant AAT in a native configuration and facilitate degradation of the misfolded protein (Flotte, 2011). Lastly, by analyzing all of the data of preclinical and clinical studies, more efficient vector systems could be designed in future to cure AAT deficiency diseases.

6. Conclusions

These viral and non-viral vectors showed advantages as well as disadvantages in curing AAT deficiency diseases. Among tested rAAV serotypes, the rAAV8 was found to be more powerful gene therapy vector for treating lungs and liver diseases. For liver diseases several approaches have been implemented like siRNA, ribozyme technology, gene repair, PNA and SDF. Their usefulness in clinical trials remain to be seen. The newly developed AAV vector that packages ds DNA looks promising for treating AAT deficiency diseases.

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8. References

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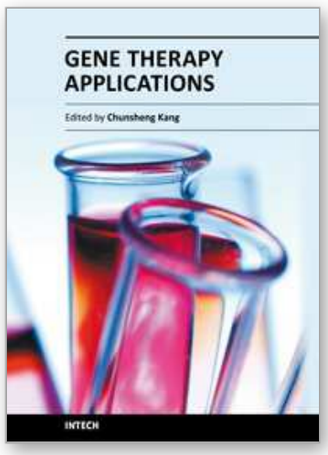

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The aim of our book is to provide a detailed discussion of gene therapy application in human diseases. The book brings together major approaches: (1) Gene therapy in blood and vascular system, (2) Gene therapy in orthopedics, (3) Gene therapy in genitourinary system, (4) Gene therapy in other diseases. This source will make clinicians and researchers comfortable with the potential and problems of gene therapy application.

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